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In vitro Anticancer Activity of *Hibiscus vitifolius* Flowers Ethyl Acetate Fraction against Hepg2 Cell Line

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Objective: The roots of *Hibiscus vitifolius* (Malavaceae) are used for the treatment of jaundice in the folklore system of medicine in India. This study is an attempt to evaluate anticancer activity of the flowers of *Hibiscus vitifolius* against Liver Cancer cell Line (HepG2).
Materials and Methods: In *vitro* cytotoxicity activity was carried out to screen cytotoxicity potency of the ethyl acetate fraction from *Hibiscus vitifolius* flower extract at different concentrations against HepG2 cell line. The MTT (methylthiazolyl diphenyl- tetrazolium bromide) assay for cell viability and markers is predictable to confirm the cytotoxicity.
Results: The ethyl acetate fraction from the flower extract of *Hibiscus vitifolius* was tested for its anticancer activity against HepG2 cell lines (liver cancer) at various concentrations by MTT assay. It was confirmed that the IC_{50} value of this sample was 150 µg/ml against Liver

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Cancer HepG2 cell line.
Conclusion: These findings suggest that the ethyl acetate fraction obtained from the flower EtOH extracts of *Hibiscus vitifolius* have the potent anticancer activity and thereby justifying its ethnopharmacological claim.

Keywords: Hibiscus vitifolius; anti-cancer activity; MTT assay; HepG2; cytotoxicity.

1. INTRODUCTION

Nature always stands as a golden mark to represent the outstanding phenomenon of symbiosis. The plants are absolutely necessary for human life. So the uses of natural products including medical plants have become vital important in primary health care especially in all the developing countries [1]. Many pharmacognostical and pharmacological investigations are carried out to identify new drugs or to find out new lead structures to develop novel therapeutic agents for the treatment of human diseases such as cancer [2-4]. Cancer is also known as a malignant tumor (or) malignant neoplasm, which is a group of diseases involving abnormal cell growth in human body with potential to invade (or) spread to other parts of the body [5]. The International Agency for Research on Cancer (IARC) estimates the incidence of mortality and prevalence from major types of cancer cell, at national level. For 184 countries of the world revealed that there were 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people were living with cancer in 2012 world wide By 2030. it is projected that there will be 26 million new cancer cases and 17 million cancer deaths per year [6-9]. Although the plant and its extracts have been extensively used in the jaundice, and associated liver damages in India [10] no literature was found on the cytotoxicity action from the flower extract of *Hibiscus vitifolius*. And also there is an increase in the use of medicinal plants and their phyto constituents in recent times. But the scarcities of scientific studies on their safety and efficacy have raised concerns in the scientific community. So there is a need to assess the potential effects of these plants. Keeping this in view, the present study has been undertaken to investigate the anticancer potential of the ethyl acetate fraction from the flower *Hibiscus vitifolius*.

2. MATERIALS AND METHODS

2.1 Plant Materials

The fresh flowers *of Hibiscus vitifolius* were collected from Z. Suthamalli, Ariyalur (Dt), Tamil Nadu, India, during the month of August and identified by Head, PG & Research Department of Botany, Periyar E.V.R. College, Trichy, Tamil Nadu.

2.2 Flower Extraction

2 kg of fresh flowers were soaked with 90% ethanol at room temperature (25° - 30℃). After 72 hrs, the ethanolic extract was filtered. This extract was fractionated with petroleum ether, diethyl ether and ethyl acetate successively. The ethyl acetate fraction was tested for anticancer activity. The dry powder obtained was dissolved in DMSO to get 50, 100, 150, 200 µg/ml concentrations.

2.3 *In vitro* Anti-cancer Activity

2.3.1 Cell line and culture

HepG2 (liver) cell lines were obtained from National Center for Cell Sciences Pune (NCCS). The cells were maintained in Minimal Essential Media (MEM) supplemented with 10% FBS (Foetal Bovine Serum), penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml of CO_2 at 37℃.

2.3.2 Reagents

MEM was purchased from Hi Media Laboratories, FBS was purchased from Cistron laboratories, Trypsin, methylthiazolyl diphenyl-tetrazolium bromide (MTT), and Dimethyl sulfoxide (DMSO) were purchased from Sisco research laboratory chemicals, Mumbai. All the other chemicals and reagents were obtained from Sigma Aldrich, Mumbai.

2.3.3 Principle of MTT assay

This is a colorimetric assay that measures the reduction of yellow 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The

cells are then solubilized with an organic solvent (eg. isopropanol) and then released. Solubilized formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.

2.3.4 *In vitro* assay for cytotoxicity activity (MTT assay)

The cytotoxicity of sample (*Hibiscus vitifolius)* on HepG2 (liver) cell line was determined by the MTT assay [11]. Cells (1×10^5/well) were plated in 1ml of medium/well in 24-well plates (Costar Corning, Rochester, NY). After 24 hours incubation, the cell reaches the confluence. Then, cells were incubated in the presence of various concentrations of the samples in 0.1% DMSO for 24 h at 37℃. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 200 µl/well (5 mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazolium bromide (MTT) phosphate -buffered saline solution was added to cells. After 4 h incubation, 0.04 M HCl/ isopropanol were added. Viable cells were determined by the absorbance at 570 nm. Measurements were performed and the concentration required for a 50% inhibition of viability (IC_{50}) was determined graphically. The absorbance at 570 nm was measured with a UV-Spectrophotometer using wells without sample containing cells as blanks. The effect of the samples on the proliferation of HepG2 (liver cell line) was expressed as the % cell viability, using the following formula:

% cell viability = A_{570} of treated cells / A_{570} of control cells

3. RESULTS

3.1 *In vitro* Assays (Cytotoxic Studies)

The ethyl acetate fraction (flower extract) effective IC_{50} value against HepG2 (liver) cell line was found to be 150 µg/ml on the MTT assay at 24 hrs. When incubated with the fraction, it induced cytotoxicity in a significant manner which implicit the damage to the membrane integrity of the cell when compared with control. The cytotoxicity was minimized in the fraction treated cells and near normal level was attained at various concentrations (50 µg/ml, 100 µg/ml, 150 µg/ml and 200 µg/ml) and maximum effect was found when treated at 200 µg/ml. From the above results, it was confirmed that the ethyl acetate fraction from *Hibiscus vitifolius* (flowers) extract at 150 µg/ml seems to offer significant protection and maintains the structural integrity of the hepatocellular membrane.

Table 1. Cell viability (%) of HepG2 cell line

Concentration (µg/ mL)	Cell viability (%)
Control	98.17±0.04
50	87.25±0.05
100	79.14±0.04
150	49.63±0.03
200	23.08±0.04
Cyclophosphamide (Positive control)180	12.37±0.03

Values are represented as mean ± SD

4. DISCUSSION

Unfavorable free radicals are generated in the body during normal metabolism and also upon exposure to environmental pollutants such as infectious agent, UV light, radiation and so on. Harmful free radicals are not neutralized by the body's primary and secondary defense mechanism on excess of unfavorable free radicals [1]. Existing clinical studies have also shown that supplemental levels of anti-oxidant vitamins (E, C and B complex) reduce, the individual risk for certain cancer [1,12-14]. Many of the medicinal plant have been found to be effective in experimental and clinical cases of cancer. Medical plants dominate immunomodulatory and anti-oxidant properties, leading to anti-cancer activity [1,15-17]. This plant has been reported to be responsible for hepatoprotective activity and oral anti-oxidant property [10]. MTT is considered to be reliable assay to determine the extent of cell viability. In the present study, the viability measured in terms of percentage was found to decrease by 98% in drug treated hepatic cell line. The cell treated with *Hibiscus vitifolius* flower extract (ethyl acetate fraction) at various concentration (50, 100, 150 and 200 µg/ml) showed protective nature of the extract act against the deleterious effects and the maximum effect was observed at 150 µg/ml (Fig. 2). The extract had an IC_{50} value of 150 µg/ml which showed cell viability 49.63% (Table 1). From the result, it clear that ethyl acetate fraction of *Hibiscus vitifolius* flower has cytotoxic effect on HepG2 cell line.

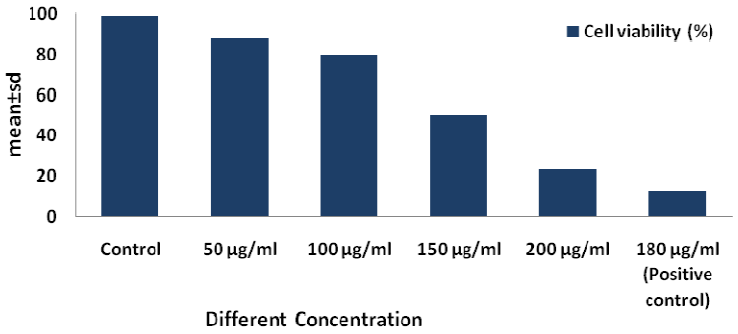

Fig. 1. Graphical reprentation of the cell viability (%) values of *Hibiscus vitifolius* (flowers) extract against HepG2 cell line

MTT assay on HepG2 cell line (Flowers extract)

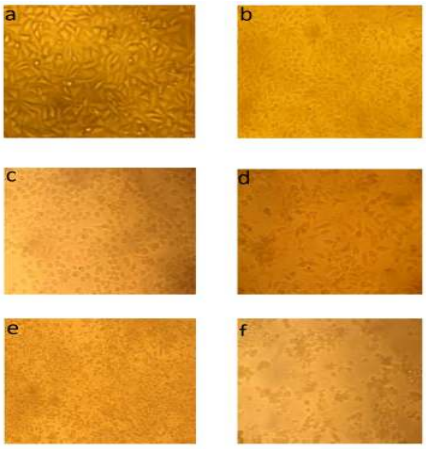

Fig. 2. A: Control cells (Untreated), b: Sample 50 µg/ml, c: Sample 100 µg/ml, d: Sample 150 µg/ml, e: Sample 200 µg/ml, f: Cyclophosphamide (Positive control) 180 µg/ml

5. CONCLUSION

The results obtained from the *in vitro* studies performed using the HepG2 cell lines reveals that the ethyl acetate fraction of the EtOH extract of *Hibiscus vitifolius* flowers has a moderate anticancer activity even though cell growth inhibition were increased when concentration of sample was increased. The IC_{50} value was more than 100 µg/ ml for the cell line studies as shown by the MTT assay method. These concentrations were able to induce apoptosis on human cancer cell lines and its anticancer activity was found to be precise. Further work is required in order to establish the identity of the chemical constituent responsible for anticancer activity. Studies are in progress in our laboratory to elucidate the molecular structure. This contributes towards the development of potent anticancer drug.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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